Journal of Oceanology and Limnology
Vol. 37 No. 3, P. 836-847, 2019
https://doi.org/10.1007/s00343-019-8138-5

Anti-oxidant and anti-inflammatory activities of ultrasonic-assistant extracted polyphenol-rich compounds from *Sargassum muticum**

YU Yuan[1], WANG Le[1], FU Xiaoting[1], WANG Lei[2], FU Xiaodan[1], YANG Min[1], HAN Zhenlian[1], MOU Haijin[1, **], JEON You-Jin[2, **]

[1] *College of Food Science and Engineering*, *Ocean University of China*, *Qingdao 266003*, *China*

[2] *Department of Marine Life Sciences*, *Jeju National University*, *Jeju 63243*, *Republic of Korea*

Received May 14, 2018; accepted in principle Jun. 23, 2018; accepted for publication Aug. 14, 2018

© Chinese Society for Oceanology and Limnology, Science Press and Springer-Verlag GmbH Germany, part of Springer Nature 2019

Abstract Polypehnol is an important, potentially bioactive component of *Sargassum muticum*. In this study, ultrasonic assisted extraction of polyphenol-rich substances was performed using a 38% ethanol solution at a solid:liquid ratio of 1:30 at 68°C for 32 min, determined by single-factor and response surface methodology (RSM) optimization. The content of polyphenol was 5.66 mg/g in the crude extract. Further extraction showed that the polyphenol mainly distributed in ethyl acetate (SKEE) and water phases (SKEW). The anti-oxidation test by electron spin resonance (ESR) spectrum showed that the SKEE had the strongest scavenging activity on DPPH (1,1-diphenyl-2-picrylhydrazyl) and alkyl radicals. SKEE was shown non-cytotoxic but could inhibit the generation of cellular ROS, showing protective effects in H_2O_2 and AAPH-induced Vero cells and UV-B irradiated HaCaT cells. SKEE also significantly inhibited the release of NO of LPS-induced RAW 264.7 cells. Therefore, the polyphenol-rich extracts in ethanol and ethyl acetate showed excellent anti-oxidant and anti-inflammatory activities, which is beneficial to the development of high-value bio-substances.

Keyword: *Sargassum muticum*; polyphenol; extraction; ESR; anti-oxidant; anti-inflammatory

1 INTRODUCTION

Sargassum muticum is a brown alga native to the Western Pacific coast. *S. muticum* is economic seaweed in East Asian countries, widely used as gelling agents and emulsifiers in food and aquaculture (Milledge et al., 2016; Puspita et al., 2017). *S. muticum* is an invasive seaweed, also widely distributed in the European Atlantic and western North American coasts (Puspita et al., 2017). Similar to other species of brown algae, *S. muticum* is rich in bioactive substances including fucoidan, alginate, phenols, unsaturated fatty acids, minerals, and vitamins (Terme et al., 2017). Therefore, algal extracts can be used to prepare functional food, aquatic feed, medicines, and cosmetic additives (Milledge et al., 2016). Polyphenol is an important secondary metabolite produced via the acetate-malonate pathway in brown seaweed (Zhang et al., 2018). Unlike the polyphenols found in terrestrial plants, the polyphenols in brown algae are polymerized by phloroglucinol repeating units. The linkages between phloroglucinol units are mainly ether or phenyl bonds. Recently, up to 150 kinds of polyphenols have been identified (Lopes et al., 2018). The molecular weight of polyphenol from *S. muticum* is reported to be 5 000–14 000 Da (Puspita et al., 2017). Similar to other brown seaweeds, its polyphenol has potential medical application value due to its excellent bioactivities, including anti-oxidant, anti-inflammatory, anti-viral, anti-cancer, and anti-wrinkle properties (Xi et al., 2015; Montero et al., 2016; Song

* Supported by the Public Science and Technology Research Funds Projects of Ocean (No. 201505022), the Shandong Province Key Research and Development Project (No. 2017YYSP003), and the Natural Science Foundation of Shandong Province (No. ZR2017MD006)

** Corresponding authors: mousun@ouc.edu.cn; youjinj@jejunu.ac.kr
YU Yuan and WANG Le contributed equally to this work.

et al., 2016).

Reactive oxygen species (ROS) are mainly comprised of chemical compounds and free radicals, such as hydrogen peroxide, nitrogen oxide, hydroxyl radicals, superoxide free radicals, and alkyl radicals (Fernando et al., 2017). External environmental factors such as air pollution, ultraviolet radiation, microbial infection, and bad living habits (smoking, excessive drinking and high calorie diet), could lead to enhanced formation of ROS in the body (Samarakoon et al., 2014), which causes oxidative stress, results in the peroxidation of functional proteins, cell membrane lipids, nucleic acids, and threatens the survival of cells and their organization (Kang et al., 2015). Inflammation is a common protective mechanism of organisms infected or attacked by bacteria, viruses, and allergens (Sanjeewa et al., 2016a; Sanjeewa et al., 2017). In a previous study, inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) were seen to be expressed, releasing nitric oxide (NO) and prostaglandin E2 (PGE2), respective in macrophages (Sanjeewa et al., 2016b). The excessive accumulation of inflammatory factors NO and PGE2 can cause cytotoxicity, resulting in pathological changes and even cancer Wijesinghe et al., 2014). It is reported that inflammatory stress strongly upregulates ROS, which directly enhances the production of tumour necrosis factor (TNF-α) and interleukin (IL), leading to oxidative stress complications (Kim et al., 2014a; Kang et al., 2015). Preparing natural polyphenols with antioxidant and anti-inflammatory properties from seaweed is conducive to the development of functional foods and medicines, therefore promoting improvement in human health.

Polyphenols are usually extracted using methanol or ethanol solutions (Kim et al., 2013), and then further purified by ethyl acetate (Kang et al., 2013). The traditional extraction method involves a large amount of solvent and time, which is not suitable for a rapid preparation of polyphenols. The aim of this study was to discover a fast and effective way to obtain polyphenol components with good bioactivity. Polyphenols from *S. muticum* were extracted using ethanol solution assisted by ultrasonication. The extraction conditions were optimized by a response surface methodology (RSM) experiment. The extract was then further separated using n-hexane, trichloro methane, and ethyl acetate. The antioxidant and anti-inflammatory activities of the samples were evaluated by ESR spectroscopy and cell culture experiments.

2 MATERIAL AND METHOD

2.1 Chemicals and reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azobis-2-methyl-propanimidamide (AAPH), lipopolysaccharide (LPS), dimethyl pyridine N-oxide (DMPO), α-(4-Pyridyl 1-oxide)-N-tert-butylnitrone (POBN), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), phosphate buffer saline (PBS, pH 7.4), dimethyl sulfoxide (DMSO), fluorescent probe 2′,7′-dichlorodihydroflurescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 The extraction of a polyphenol-based substance

Sargassum muticum was rinsed with clean water repeatedly to remove the impurities on the surface and then oven-dried at 40°C for 24 h. The dried seaweed was smashed and sieved through a 0.18-mm mesh. The extraction of the polyphenol-rich substance from *S. muticum* was performed in an ultrasonic cleaning bath, with an output power and irradiation frequency at 200 W and 40 kHz, respectively. The effects of ethanol concentration, ultrasonic time, ultrasonic temperature and liquid-material ratio on the extraction yield of polyphenol were investigated. One variable was studied in each experiment while the other factors were kept constant. Total polyphenol content was determined according to the Folin-Ciocalteu method (Singleton and Rossi, 1965).

2.3 Optimizing the process of extraction by RSM

RSM was used to optimize the extraction parameters of BBD with three factors at three levels based on single-factor experiments. Ethanol concentration, ultrasonic time, and ultrasonic temperature were selected for the optimization of polyphenol extraction. Using the Boxe-Behnken method, the whole design matrix was composed of 17 experiments in random order. The design of the experiments is given in Table 1. For the three variables, the experimental data of the system was fitted to the following quadratic equation (Qiao et al., 2009):

$$Y = \sum \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=3}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j,$$

where Y is the dependent variable and X_i, X_j are the independent variables; β_0, β_i β_{ii}, and β_{ij} are the regression coefficients of the independent variables that were estimated by the model for intercept, linear, quadratic and interaction terms, respectively. Design-Expert 8.0 was used for statistical analysis of variance for each response and for predicting the optimal conditions for the extraction.

2.4 The fractionation of biological substances

After ethanol solution extraction, n-hexane, trichloro methane and ethyl acetate were used to separate biological substances, respectively. First, n-hexane was used to extract and the upper layer was collected, denoted as SKEH. Then, trichloro methane was applied and the lower layer was collected, denoted as SKEC. Lastly, ethyl acetate was used and the upper layer was collected, denoted as SKEE. The remaining water layer was denoted as SKEW. The content and distribution of polyphenol in each phase were determined by the FC method.

2.5 Electron spin resonance (ESR) experiment

In this study, the scavenging activity of ethanol-extracted samples on the DPPH, hydroxyl radical and an alkyl radical was determined on a JES-FA 200 ESR spectrometer (JEOL Ltd., Tokyo, Japan).

DPPH solution was prepared using methanol at a final concentration of 60 μmol/L. Then, 60 μL of DPPH and 60 μL of extracted samples (final concentration of 100–500 μg/mL) were mixed. After 2 min of reaction at 25°C, the reaction liquid was transferred to a 50-μL capillary and measured in the ESR instrument. The conditions were as follow: power 1 mW, amplitude 500, modulation width 0.8 mT, sweep field width 10 mT, scanning time 30 s, the time constant 0.03 s and temperature 25°C.

The alkyl radical scavenging reaction system contained 20 μL of distilled water, 20 μL of extracted sample (final concentration 50–250 μg/mL), 20 μL of AAPH solution (40 mmol/L) and 20 μL of POBN solution (40 mmol/L). After mixing, the reaction was finished at 37°C for 30 min, then the reaction liquid was transferred to and measured in the ESR instrument. The determination conditions of ESR were as follows: power 1 mW, amplitude 1 000, modulation width 0.2 mT, sweep field width 10 mT, scanning time 30 s and temperature 25°C.

The hydroxyl radicals scavenging reaction system contained 20 μL of extracted samples (final concentration 50–250 g/mL), 20 μL of DMPO (0.3 mol/L), 20 μL of $FeSO_4$ (10 mmol/L) and 20 μL of H_2O_2 (10 mmol/L). After 2.5 min of reaction at room temperature, the reaction liquid was transferred to and measured in the ESR instrument. The determination conditions of ESR were: power 1 mW, amplitude 200, modulation width 0.1 mT, sweep field width 10 mT, scanning time 30 s, and temperature 25°C.

Table 1 Response and experiment design of polyphenol extracted from *S. muticum*

Run	Experimental factor			Extraction yield (mg/g)
	Ethanol concentration (%)	Ultrasonic time (min)	Ultrasonic temperature (°C)	
1	50(1)	15(-1)	70(0)	5.24
2	50(1)	30(0)	80(1)	5.37
3	40(0)	30(0)	70(0)	5.63
4	30(-1)	30(0)	80(1)	5.35
5	30(-1)	30(0)	60(-1)	5.54
6	40(0)	30(0)	70(0)	5.71
7	40(0)	30(0)	70(0)	5.69
8	40(0)	45(1)	60(-1)	5.38
9	40(0)	15(-1)	80(1)	5.22
10	30(-1)	15(-1)	70(0)	5.18
11	40(0)	15(-1)	60(-1)	5.26
12	40(0)	30(0)	70(0)	5.74
13	40(0)	45(1)	80(1)	5.23
14	50(1)	30(0)	60(-1)	5.32
15	50(1)	45(1)	70(0)	5.28
16	40(0)	30(0)	70(0)	5.66
17	30(-1)	45(1)	70(0)	5.46

The number in the bracket (1, 0, -1) represents the corresponding level codes of selected factors.

2.6 Cell experiment

2.6.1 Cell culture

Vero cells were cultured in the RPMI-1640 medium. HaCaT cells and RAW 264.7 cells were cultured in the DMEM medium. Each medium contained 10% heat inactivated FBS, 100 μg/mL of streptomycin and 100 U/mL penicillin. Cells were cultured at 37°C in an incubator and with a CO_2 concentration of 5%.

2.6.2 Cell cytotoxicity test

The cytotoxicity of samples to Vero, HaCaT and RAW 264.7 were determined by MTT assay method. The cells were seeded in 96-well plates with a

concentration of 1×10^5/mL, cultured at 37°C for 24 h. The cells were then treated with 10 μL of sample. The final concentration of the samples was 25, 50, 100, 200 and 400 μg/mL. Then, 10 μL of PBS buffer (pH 7.4) was added to the blank group. Cells were cultured at 37°C for another 24 h and 50 μL of MTT solution (2 mg/mL) was added with a reaction time was 3 h. After the medium was removed, 150 μL of DMSO was added and shaken overnight. The results were measured at 540 nm. The cell viability of the blank group was regarded to be 100%.

2.6.3 Anti-oxidant effect of samples on AAPH and H_2O_2 induced oxidative stress

Vero cells were seeded with a concentration of 1×10^5/mL in 96 well plates and cultured at 37°C for 24 h. After culturing, the cells were treated with 10 μL of samples (final concentration 25, 50, 100, 200 μg/mL) for 1 h at 37°C. The cells were then treated with 10 μL H_2O_2 (1 mmol/L) and AAPH (10 mmol/L), respectively. A part of the cells was stored for 1 h at 37°C, and 10 μL of DCFH-DA solution (500 μg/mL) was added to determine the amount of cellular ROS. Fluorescent intensity was determined at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Other cells were cultured for another 24 h at 37°C, and their cell viability was measured by MTT method. The cell viability of non-AAPH or H_2O_2 treated group was regarded to be 100%.

2.6.4 Anti-oxidant effect of samples on UV-B induced oxidative stress

HaCaT cells were seeded with a concentration of 1×10^5/mL in 24-well plates and cultured for 24 h at 37°C. And then cells were treated with 25 μL of samples (final concentration 25, 50, 100, 200 μg/mL) for 30 min. After sample treatment, the cells were washed with PBS and irradiated by UV-B with an energy of 30 mJ/cm^2. One part of the cells were treated with 25 μL of DCFH-DA solution, the amount of cellular ROS was measured after 30 min reaction. Other cells were cultured for another 24 h and the cell viability was determined by MTT method. The cell viability of non-UVB irradiated group was regarded to be 100%.

2.6.5 Anti-inflammatory effect of samples on LPS induced inflammatory stress

RAW 264.7 cells were seeded at a concentration of 1×10^5/mL in 24-well plate and then cultured for 24 h at 37°C. The cells were treated with 25 μL of samples (final concentration 12.5–200 μg/mL). Then, 25 μL of PBS (pH 7.4) was added to the blank group. After 1 h of treatment, 25 μL of LPS solution (final concentration 1 μg/mL) was added and the cells were cultured for another 24 h. The cell viability was determined by MTT as described above. The supernatant (100 μL) was used to determine the amount of NO by the Griess method, whereby 100 μL of Griess reagent was added. The results were measured at 540 nm after 10 min of reaction. The release of NO in the LPS treatment group was set to 100%.

2.7 Statistical analysis

All the data were performed in triplicate. The data statistics were completed by SPSS 18.0 software. All data are expressed as means±standard deviation (SD). The cell viability and ROS, the NO amounts in the cell experiment were analyzed by one-way ANOVA combined with Tukey's *B*-test. $P<0.05$ was considered significant and $P<0.01$ very significant.

3 RESULT

3.1 Single-factor extraction experiments

The results of single-factor extraction experiments are shown in Fig.1. The yield of polyphenol increased along with the increase of ethanol concentration from 20% to 40% and decreased at the concentration higher than 40%. When the ethanol concentration was 40%, the yield of polyphenol reached a maximum of 5.66±0.14 mg/g.

When the extraction time was 30 min, the yield of polyphenol reached a maximum value of 5.69±0.08 mg/g. The extraction yield began to decrease after 30 min.

The extraction yield of polyphenol increased with the increase of ultrasonic temperature. However, when the temperature exceeded 78°C, the ethanol became volatilized, which was not conducive to the extraction of polyphenol. Based on this, the extraction temperature was set at 70°C for the following optimization experiment.

The yield of polyphenol was found to increase gradually when the ratio of liquid to material ranged from 10 mL/g to 30 mL/g. There was a maximum of 5.75±0.10 mg/g at 30 mL/g, and when the ratio of liquid to material exceeded 30 mL/g, the yield of polyphenol decreased.

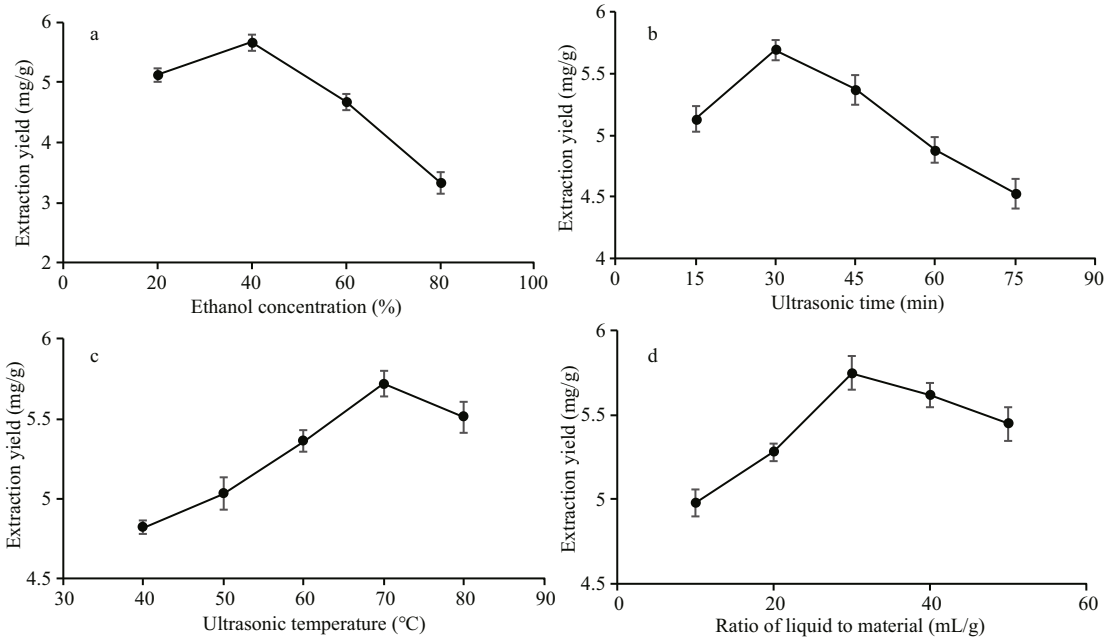

Fig.1 Effects of extraction conditions on the extraction yield of polyphenol

a. ethanol concentration; b. ultrasonic time; c. ultrasonic temperature; d. ratio of liquid to the material.

3.2 Extraction optimization using RSM

3.2.1 Fitting of the second-order polynomial equation and statistical analysis

Based on the results of the single-factor experiments, ethanol concentration (30%–50%), ultrasonic time (15–45 min) and ultrasonic temperature (60–80°C) were selected for the optimization of the polyphenol yield. A group of 17 experiments was executed in analogy with the Box-Behnken design. The variables of actual and coded levels as well as of polyphenol yield are shown in Table 1. A quadratic equation was used to establish a statistical model to confirm the optimum conditions and the response of the combined factors. By using multiple regression analysis on the experimental data, the yield of polyphenol was obtained using the following quadratic equation:

$$Y=5.15-0.16A+0.11B+0.58C-0.06AB+0.06AC-0.027BC-0.14A^2-0.26B^2-0.15C^2,$$

where Y was the predicted response; A, B, and C were the ethanol concentration (%), ultrasonic time (min) and ultrasonic temperature (°C)

The variance analysis and linear coefficient of the regression model, the coefficient of the two-item coefficient interaction and its significance test results are presented in Table 2. It could be seen from the analysis of table variance that the large F-value (35.67) and small P-value (<0.000 1) indicate that the experimental design model was satisfactory and that the effect of the different factors on the yield of polyphenol was significant. The lack of fit (0.470 6>0.05) was not significant, indicating that the fitting degree of the model was successful. The adjoint determination coefficient (R^2_{Adj}=0.951 2) and the

Table 2 ANOVA of the quadratic polynomial model and significance test

Source	Sum of Squares	df	Mean Square	F Value	P-value Prob>F	Significance
Model	0.59	9	0.066	35.67	<0.000 1	[b]
A	0.023	1	0.023	12.31	0.009 9	[b]
B	0.011	1	0.011	5.95	0.044 8	[a]
C	0.084	1	0.084	45.67	0.000 3	[b]
AB	0.014	1	0.014	7.79	0.026 9	[a]
AC	0.014	1	0.014	7.79	0.026 9	[a]
BC	0.003	1	0.003	1.64	0.241 7	
A^2	0.079	1	0.079	42.58	0.000 3	[b]
B^2	0.28	1	0.28	153.03	<0.000 1	[b]
C^2	0.10	1	0.10	54.17	0.000 2	[b]
Residual	0.013	7	0.001 8			
Lack of fit	0.005 6	3	0.001 9	1.02	0.470 6	
Pure error	0.007 3	4	0.001 8			
Cor total	0.61	16				

C.V. %=0.79; R^2=0.978 7; Adj R^2=0.951 2; Pred R^2=0.832 8; Adep Precision=14.659. A: ethanol concentration (%); B: ultrasonic time (min); C: ultrasonic temperature (°C). [a] P<0.05 (significant); [b] P<0.01 (highly significant).

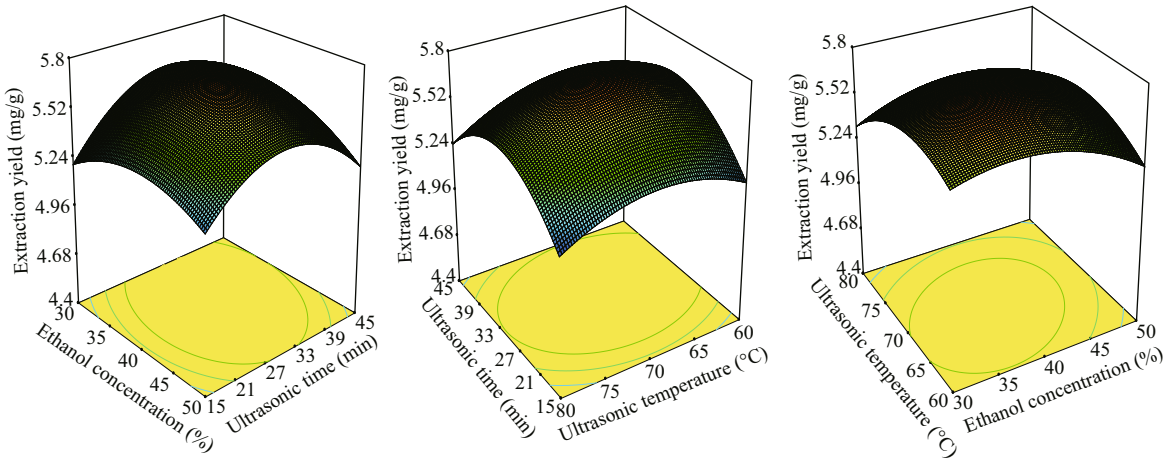

Fig.2 Response surface plots of variable parameters on the yield of polyphenol

determination coefficient (R^2=0.978 7) of the model indicate that the model had a small error and high accuracy. Therefore, the model suggested that the change of response value could be used to analyze and pre-test the process parameters of ultrasonic assisted extraction of polyphenol from *S. muticum*.

The interaction of the ethanol concentration, ultrasonic time, and ultrasonic temperature parameters were significant (P<0.05) in the model, while the interaction of ultrasonic time and the ultrasonic temperature was not significant. These data clearly supported the conclusion that ethanol concentration, ultrasonic time, and the ultrasonic temperature had a significant impact on the extraction yield.

3.2.2 Analysis and optimization of RSM

The three-dimensional diagrams of the RSM model for ultrasound-assisted polyphenol extraction are shown in Fig.2. When one factor was fixed at a centered level, the interaction between other factors could be reflected in the diagrams. It was found that when the independent variable was at a low level, the extraction yield increased gradually. However, when the independent variable exceeded a certain value, the extraction yield decreased with the increase of the independent variable.

To further confirm the suitability of the above-mentioned equation, a validation experiment was performed. The extraction conditions were adjusted to an ethanol concentration of 38%, ultrasonic time of 32 min and ultrasonic temperature of 68°C. We found that the resulting value (5.66 mg/g) was approximately the same as the maximum predicted value (5.70 mg/g), clearly demonstrated the aptness of the model under these conditions. As a result, the model could be considered accurate and reliable for predicting the extraction of polyphenol.

Table 3 Content of polyphenol after fractionation by n-hexane, trichloro methane and ethyl acetate

	SKE	SKEH	SKEC	SKEE	SKEW
Sample weight (mg)	547.33	76.57	312.9	9.95	21.63
Polyphenol content (%)	2.89	0.45	0.23	36.48	41.70
Yield of polyphenol (mg)	15.80	0.35	0.72	3.63	9.02

SKE: ethanol extract; SKEH: n-hexane extraction layer; SKEC: trichloro-methane extraction layer, SKEE: ethyl acetate extraction layer; SKEW: water layer.

3.3 The fractionation of biological substances

The biological substances were extracted from *S. muticum* according to the optimized extraction condition. The ethanol extraction contained various components, including polyphenols, pigments, polysaccharides, and proteins, which showed different biological activities. In this study, n-hexane, trichloro methane, and ethyl acetate were used to separate the ethanol extraction to obtain preliminary purified biological substances. Table 3 shows that the polyphenol content in ethanol extract from 3 g *S. muticum* powder was 15.8 mg. After extraction by n-hexane, trichloro methane, and ethyl acetate, polyphenols were found mainly distributed in ethyl acetate and water. The content of polyphenols in SKEE phase was 36.5%, accounting for 23.0% of the total polyphenols, while the content of polyphenols in the SKEW phase was 41.7%, accounting for 57.1% of the total polyphenols. The content of polyphenols in SKEC was low, but the quantity of dry matter was large due to pigments. The proportion of polyphenols in the SKEH phase was the smallest.

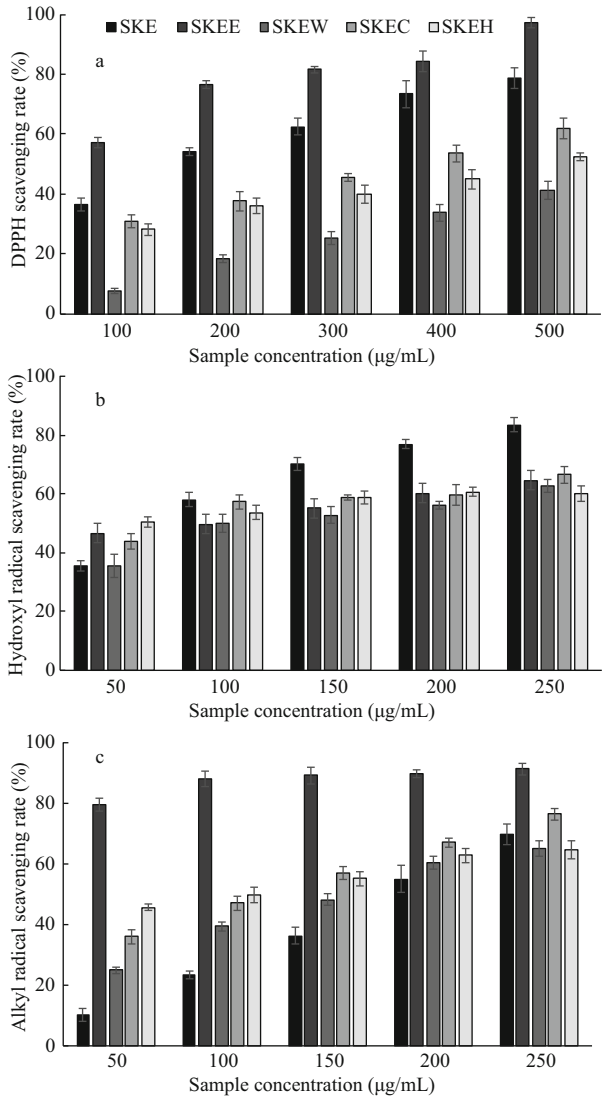

Fig.3 ESR experiments of bio-substances

a. DPPH scavenging activity; b. hydroxyl radical scavenging activity; c. alkyl radical scavenging activity.

3.4 ESR experiments

The five kinds of biological substances (SKE, SKEE, SKEW, SKEH, SKEC) all showed a dose-dependent increase in DPPH scavenging (Fig.3a). The scavenging activity of SKEE was the strongest. The scavenging rate was 57.2% at 100 μg/mL. When the concentration was increased to 200 μg/mL, the scavenging rate reached 76.7%. The scavenging rate of SKE was 36.5% and 78.6% at 100 and 500 μg/mL, respectively. For SKEH, SKEC, and SKEW, the scavenging rates were 52.5%, 62.1%, and 41.3% at 500 μg/mL, respectively, which is lower than those of SKE and SKEE.

All the five substances showed excellent scavenging activity of the hydroxyl radical. As shown in Fig.3b, the scavenging rate of SKE was 83.6% at 250 μg/mL, while SKEE, SKEW, SKEH and SKEC showed scavenging rates of 64.6%, 62.8%, 60.1%, and 66.6% at a concentration of 250 μg/mL, respectively.

All five substances showed a dose-dependent increase in alkyl radical scavenging, among which SKEE showed the highest scavenging activity (Fig.3c). The scavenging rate reached 79.9% and 91.4% at 50 μg/mL and 250 μg/mL, while those from SKE, SKEW, SKEC and SKEH were 10.4%, 25.1%, 36.3%, and 45.8% at 50 μg/mL, 69.9%, 65.1%, 76.5%, and 64.7% at 250 μg/mL, respectively.

The half scavenging concentrations of DPPH, hydroxyl radicals and alkyl radicals (IC_{50}) of the five substances are listed in Table 4. The results show that SKEE had the strongest scavenging activity on DPPH and alkyl radicals.

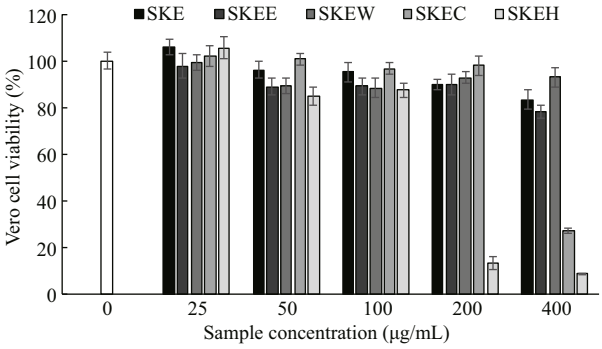

Fig.4 Vero cell viability after sample treatment

The cell viability of the non-sample treated group was regarded to be 100%.

Table 4 The half scavenging concentration (IC_{50}, μg/mL) of five substances on DPPH, hydroxyl radicals and alkyl radicals

	SKE	SKEE	SKEW	SKEH	SKEC
DPPH	192.8	87.4	598.8	468.6	356.2
Hydroxyl radical	85.0	92.8	137.4	63.3	73.7
Alkyl radical	186.1	31.3	161.1	93.7	115.9

SKE: ethanol extract; SKEH: n-hexane extraction layer; SKEC: trichloromethane extraction layer, SKEE: ethyl acetate extraction layer; SKEW: water layer.

3.5 Sample cytotoxicity test

Before testing the bio-activity of samples, it was necessary to measure the cytotoxicity effect of samples. The results are shown in Fig.4. For SKE, when the concentration was less than 200 μg/mL, the cell viability remained at approximately 90% and proved to be non-toxic to Vero cells. When the sample concentration reached 400 μg/mL, cell viability

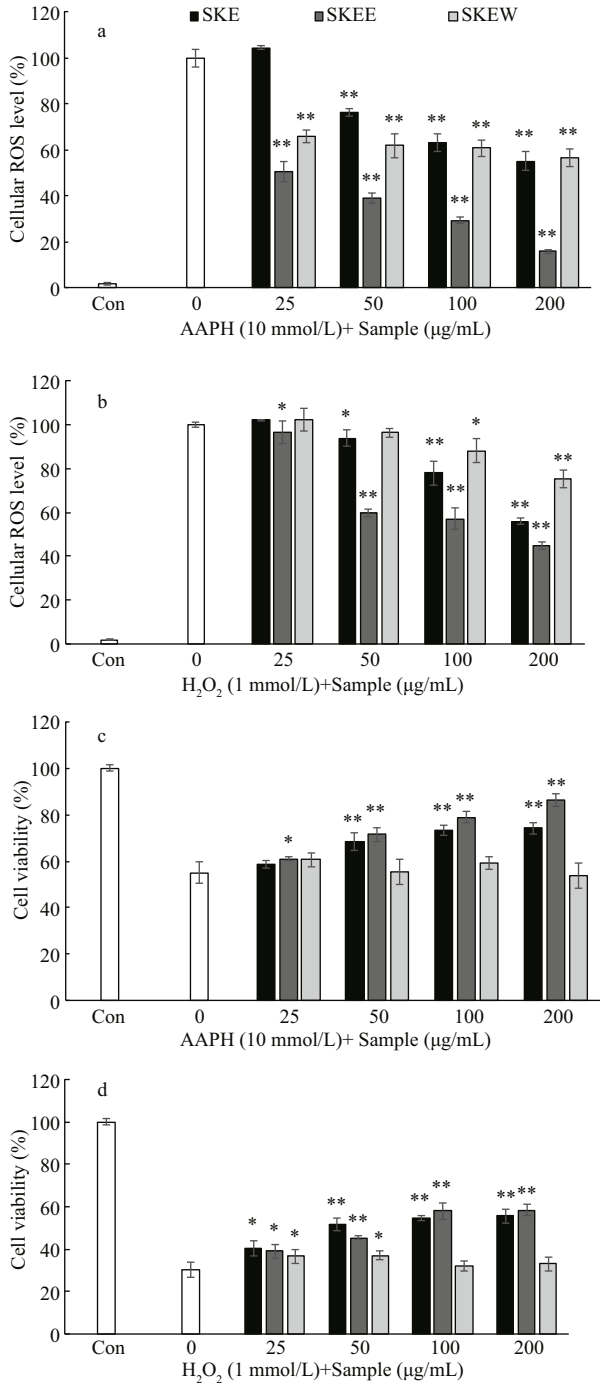

Fig.5 Anti-oxidant effect of samples on Vero cells

a. effect of samples on AAPH induced cellular ROS secretion; b. effect of samples on H_2O_2 induced cellular ROS secretion; c. cytoprotective effect of samples on AAPH induced cells; d. cytoprotective effect of samples on H_2O_2 induced cells. Experiments were performed in triplicate and the means with superscript are significantly different with a non-sample treated group (*P<0.05 and **P<0.01).

decreased to 83.4%. Similarly, SKEE showed non-toxic effects at a concentration of 200 μg/mL, while 400 μg/mL of SKEE lead to a drop in the cell viability dropped down to 78.5%. SKEW proved to be non-toxic to cells, and even when its concentration reached 400 μg/mL, the cell survival rate was 93.1%. SKEH and SKEC showed significant toxicity to cells at 200 μg/mL. Therefore, the following experiments only used the non-toxic samples of SKE, SKEE, and SKEW.

3.6 Anti-oxidation effect of samples on Vero cells

AAPH is an inducer of alkyl radicals. The increasing content of cellular ROS induced by AAPH can cause serious oxidative damage to cells. Figure 5a shows that the cellular ROS decreased after sample treatment. The SKEE sample showed the strongest antioxidant activity. Moreover, when the concentration was at 200 μg/mL, SKEE effectively inhibited the production of ROS, and AAPH-induced ROS production decreased to 15.9%. For SKE and SKEW, the cellular ROS decreased to 55.1% and 56.7%, respectively, which were lower than that of SKEE.

H_2O_2 is able to penetrate the cell membrane and react with Fe^{2+} in the cell. The production of hydroxyl radical can damage cells. As shown in Fig.5b, the secretion of cell ROS showed a dose-dependent decrease, among which SKEE showed the strongest antioxidant activity. When the sample concentration was 200 μg/mL, cellular ROS production was found to decrease to 44.8%. For SKE and SKEW treated cells, the cellular ROS decreased to 55.9% and 75.3%, respectively.

Upon the induction of AAPH, the cell viability significantly decreased. Figure 5c shows the viability of Vero cells treated by AAPH decreased to 55.1%. SKE and SKEE protected cells and the cell viability showed a dose-dependent increase. The oxidative damage of cells decreased after sample treatment. When the concentration of SKE and SKEE was 200 μg/mL, the survival rate of Vero cells increased to 74.1% and 86.5%, respectively. The protective effect of SKEE was stronger than that of SKE. However, the SKEW sample did not show a significant increase in cell viability. When the concentration was 200 μg/mL, the cell survival rate was only 53.8%.

The viability of Vero cells treated with H_2O_2 sharply decreased to 30.5%, indicating that hydroxyl radicals are damaging to Vero cells. As shown in Fig.5d, the cell viability also showed a dose-dependent increase in the treatment of SKE and SKEE. At the concentration of 200 μg/mL, the cell viability rate increased to 55.6% and 58.5%, respectively. For the SKEW group, cell viability did not change significantly; the cell viability was only 33.1% at 200 μg/mL.

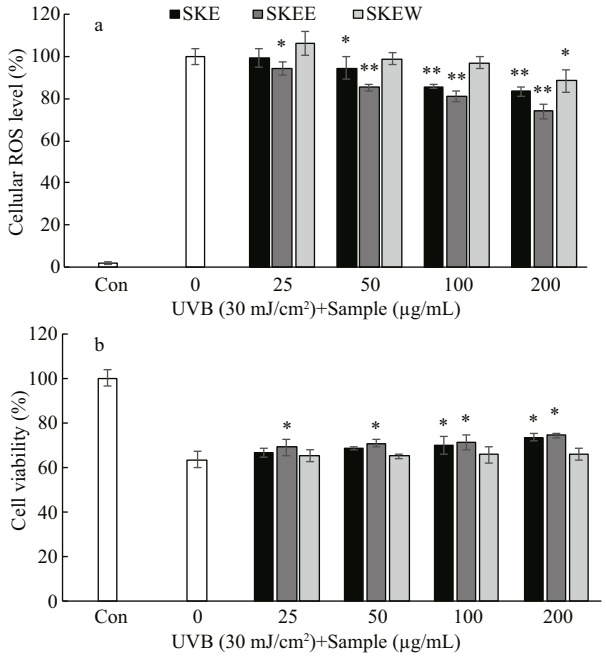

Fig.6 Anti-UVB effect of samples on HaCaT cells

a. effect of samples on UV-B induced cellular ROS secretion; b. cell viability of HaCaT cells after sample treatment. Means with superscript are significantly different with a non-sample treated group (**P*<0.05 and ***P*<0.01).

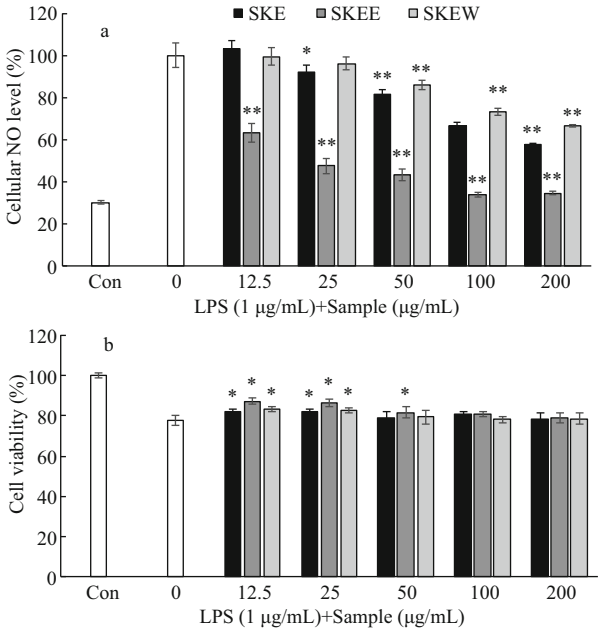

Fig.7 Anti-inflammatory effect of samples on RAW 264.7 cells

a. effect of samples on LPS induced cellular NO secretion; b. cell viability of LPS induced RAW 264.7 cells after sample treatment. Means with superscript are significantly different with a non-sample treated group (**P*<0.05 and ***P*<0.01).

3.7 Anti-ultraviolet effect of samples on HaCaT cells

The cellular ROS produced by normal HaCaT cells was in a low range, while the 30 mJ/cm^2 of UV-B irradiation caused oxidative damage to cells, releasing large amounts of ROS. When cells were treated with the extracted samples, the ROS secretion of HaCaT cells showed a dose-dependent decrease (Fig.6a). When the treatment dose of SKE, SKEE, and SKEW was 200 μg/mL, the cellular ROS secretion of HaCaT cells decreased to 83.5%, 73.9%, and 88.6%, respectively. SKEE showed the strongest anti-UV effect.

The protective effect of samples on UV irradiated cells is shown in Fig.6b. The UV-B irradiation damaged HaCaT cells and cell viability decreased to 63.6%. With the treatment of SKE, SKEE, and SKEW, the viability of HaCaT cells increased to 73.6%, 74.4%, and 66.1%, respectively, showing that all three samples had a protective effect on the cells, in which SKEE showed the strongest anti-UVB irradiation activity.

3.8 Anti-inflammatory effect of samples on RAW 264.7 cells

LPS can induce inflammatory stress in cells. Large amounts of NO are produced during the anti-inflammatory reaction, which directly reflects the degree of inflammatory stress. The anti-inhibitory effect of the three extracted samples is shown in Fig.7a. After treatment with the extracted samples, NO produced by RAW 264.7 cells showed a dose-dependent decrease. SKEE showed excellent anti-inflammatory activity, and the cellular NO decreased to 52.3% at a concentration of 25 μg/mL. When the concentration increased to 100 μg/mL, NO production decreased to 34.0%, which was close to that of the blank group. For SKE and SKEW, when the sample concentration was 200 μg/mL, the cellular NO decreased to 57.8% and 66.7%, respectively. This shows that SKEE has stronger anti-inflammatory activity than SKE and SKEW.

As shown in Fig.7b, the viability of RAW 264.7 cells decreased to 77.7% after LPS treatment. This suggests that the inflammatory stress induced by LPS caused damage to the cells. The cell viability of RAW 264.7 cells treated by samples increased to 83.2%, 87.2%, and 82.3% with 25 μg/mL of SKE, SKEE and SKEW treatment, respectively. This suggests that the protective effect of SKEE on LPS damaged cells is better than that of SKEW and SKE.

4 DISCUSSION

In the previous reports, a polyphenol extracted by methanol solution from *S. muticum* collected from

Norway and Portugal showed the highest DPPH scavenging ability among the tested seaweeds, with an IC_{50} value between 0.41 to 0.46 mg/mL (Tanniou et al., 2014). Polyphenol extracted by enzymes from *S. muticum* showed an IC_{50} value between 0.6–2.2 mg/mL on DPPH scavenging (Puspita et al., 2017). Electron spin resonance (ESR) is suitable for the rapid analysis of free radicals, and the peak of DPPH, hydroxyl radicals and alkyl radicals show significant differences in ESR. In general, the height of the peak is correlated positively with the content of the free radicals. In this study, ESR analysis of *S. muticum* extraction showed that the ethyl acetate phase (SKEE) possessed the highest scavenging ability of DPPH and alkyl radicals. The results indicated that ethyl acetate-soluble polyphenol exhibited stronger radicals scavenging activity than water-soluble polyphenol.

Diseases caused by metabolic disorders, such as skin diseases, liver injury, Alzheimer's disease, cardiovascular, cancer, and physiological phenomena, such as aging and obesity, are all closely related to the over production of ROS (Kim et al., 2014a; Ko et al., 2014). In the food industry, lipid peroxidation and rancidity induced by ROS lead to food safety hazards (Ko et al., 2013). Although the synthetic antioxidants butyl hydroxy anisole, propyl gallate, and butylated hydroxytoluene have strong antioxidant activities, they are food additives and cannot be consumed in large quantities (Kang et al., 2014). Therefore, research on natural antioxidants from *S. muticum* is important in the food and pharmaceutical industry. Namvar et al. (2013) reported that polyphenol from *S. muticum* showed dose-dependent reducing power and inhibition of breast cancer cells. In our study, SKE and SKEE were not found to have a toxic effect on Vero cells at concentrations of 200 μg/mL and showed excellent anti-oxidant activity. Especially for SKEE, the AAPH and H_2O_2 induced cellular ROS sharply decreased to 15.8% and 44.8%, respectively. The viability of Vero cells increased by 42.2% and 92.4% compared to the control AAPH and H_2O_2 treatment groups, respectively. This suggests that due to the excellent scavenging activity on radicals, SKEE significantly protected Vero cells, and could be developed as a natural anti-oxidant product.

UV-B irradiation destroys the oxidative balance on the skin's surface, resulting in skin DNA damage (Kang et al., 2015; Kim et al., 2017). Heo et al. (2009) and Ko et al. (2011) both reported that polyphenol from brown seaweed *Ecklonia cava* could inhibit the release of cellular ROS and protect UV-B irradiated HaCaT cells. Similarity, in our study, SKE and SKEE extracted from *S. muticum* both decreased the HaCaT cellular ROS level. When SKE and SKEE treatment concentration was 200 μg/mL, the UV-irradiated cell viability increased 15.9% and 17.0%, respectively, showing that both SKE and SKEE showed protective effects on UV-irradiated HaCaT cells.

When RAW 264.7 cells are treated with LPS, inflammatory stress will occur. The NF-κB pathway will be activated and transcription factor iNOS will be up-regulated, results to the release of NO (Fernando et al., 2016). According to the reports of *S. muticum* extraction, 25 μg/mL of dichloro methane fraction could inhibit the LPS-induced NO product by 86% (Yoon et al., 2010), the main component apo-9-fucoxanthinone showed dose-dependent decrease of NO release, the expression of factor TNF-α and IL-4 were also inhibited (Yang et al., 2013; Han et al., 2016). Kim et al. (2014b) reported that the ethyl acetate fragment of brown seaweed *E. cava* showed excellent inhibition effects on iNOS expression and NO production. In our study, trichloro methane fraction (SKEC) had toxicity to cells, which limited its application. However, ethyl acetate fragment (SKEE) showed non-toxic, the cellular NO production under 200 μg/mL of SKEE treatment was similar to that of a non-LPS treatment group. This suggests that SKEE inhibit the LPS-induced iNOS production, resulting in a lower cellular NO release in RAW 264.7 cells. SKEE also protected LPS treated cells. When its concentration was 25 μg/mL, the viability of RAW 264.7 cell increased 11.3%. This result confirms the anti-inflammatory effects of SKEE.

In our study, although SKEW contains a relatively high content of polyphenols, its activity was not ideal. This may be due to the structure and properties of polyphenols dissolved in ethyl acetate and water being different. In a further study, the structures of the polyphenols will be elucidated for the better understanding of the relationship between the structure and bioactivity.

5 CONCLUSION

After the optimization of ethanol extraction and the fractionation of the bio-substances, polyphenol was obtained from *S. muticum* that showed excellent anti-oxidant and anti-inflammatory activities, among which SKEE showed the best anti-oxidant and anti-inflammatory effects, followed by SKE and SKEW, while SKEH and SKEC were found to be toxic to cells. The development of polyphenol-rich substance

is of high value due to their potential utilization in functional food, medicine, and cosmetics.

6 DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

7 ACKNOWLEDGEMENT

The authors thank the support from China Scholarship Council (National Scholarship for studying abroad).

References

Fernando I P S, Nah J W, Jeon Y J. 2016. Potential anti-inflammatory natural products from marine algae. *Environmental Toxicology and Pharmacology*, **48**: 22-30.

Fernando I P S, Sanjeewa K K A, Samarakoon K W, Lee W W, Kim H S, Kim E A, Gunasekara U K D S S, Abeytunga D T U, Nanayakkara C, de Silva E D, Lee H S, Jeon Y J. 2017. FTIR characterization and antioxidant activity of water soluble crude polysaccharides of Sri Lankan marine algae. *Algae*, **32**(1): 75-86.

Han S C, Kang N J, Yoon W J, Kim S, Na M C, Koh Y S, Hyun J W, Lee N H, Ko M H, Kang H K, Yoo E S. 2016. External application of Apo-9'-fucoxanthinone, isolated from *Sargassum muticum*, suppresses inflammatory responses in a mouse model of atopic dermatitis. *Toxicological Research*, **32**(2): 109-114.

Heo S J, Ko S K, Cha S H, Kang D H, Park H S, Choi Y U, Kim D, Jung W K, Jeon Y J. 2009. Effect of phlorotannins isolated from *Ecklonia cava* on melanogenesis and their protective effect against photo-oxidative stress induced by UV-B radiation. *Toxicology in Vitro*, **23**(6): 1 123-1 130.

Kang M C, Kang S M, Ahn G, Kim K N, Kang N, Samarakoon K W, Oh M C, Lee J S, Jeon Y J. 2013. Protective effect of a marine polyphenol, dieckol against carbon tetrachloride-induced acute liver damage in mouse. *Environmental Toxicology and Pharmacology*, **35**(3): 517-523.

Kang M C, Kim S Y, Kim E A, Lee J H, Kim Y S, Yu S K, Chae J B, Choe I H, Cho J H, Jeon Y J. 2015. Antioxidant activity of polysaccharide purified from *Acanthopanax koreanum* Nakai stems *in vitro* and *in vivo* zebrafish model. *Carbohydrate Polymers*, **127**: 38-46.

Kang M C, Kim S Y, Kim Y T, Kim E A, Lee S H, Ko S C, Wijesinghe W A J P, Samarakoon K W, Kim Y S, Cho J H, Jang H S, Jeon Y J. 2014. *In vitro* and *in vivo* antioxidant activities of polysaccharide purified from aloe vera (*Aloe barbadensis*) gel. *Carbohydrate Polymers*, **99**: 365-371.

Kim E A, Lee S H, Ko C I, Cha S H, Kang M C, Kang S M, Ko S C, Lee W W, Ko J Y, Lee J H, Kang N, Oh J Y, Ahn G, Jee Y H, Jeon Y J. 2014a. Protective effect of fucoidan against AAPH-induced oxidative stress in zebrafish model. *Carbohydrate Polymers*, **102**: 185-191.

Kim M J, Hyun K H, Hyun J H, Im S, Sim J, Lee N H, Hyun C G. 2017. Anti-Melanogenic Activities of *Sargassum muticum* via MITF downregulation. *Oriental Journal of Chemistry*, **33**(4): 1 589-1 594.

Kim S M, Kang S W, Jeon J S, Jung Y J, Kim W R, Kim C Y, Um B H. 2013. Determination of major phlorotannins in *Eisenia bicyclis* using hydrophilic interaction chromatography: seasonal variation and extraction characteristics. *Food Chemistry*, **138**(4): 2 399-2 406.

Kim S Y, Kim E A, Kang M C, Lee J H, Yang H W, Lee J S, Lim T I, Jeon Y J. 2014b. Polyphenol-rich fraction from *Ecklonia cava* (a brown alga) processing by-product reduces LPS-induced inflammation *in vitro* and *in vivo* in a zebrafish model. *Algae*, **29**(2): 165-174.

Ko J Y, Kim E A, Lee J H, Kang M C, Lee J S, Kim J S, Jung W K, Jeon Y J. 2014. Protective effect of aquacultured flounder fish-derived peptide against oxidative stress in zebrafish. *Fish & Shellfish Immunology*, **36**(1): 320-323.

Ko J Y, Lee J H, Samarakoon K, Kim J S, Jeon Y J. 2013. Purification and determination of two novel antioxidant peptides from flounder fish (*Paralichthys olivaceus*) using digestive proteases. *Food and Chemical Toxicology*, **52**: 113-120.

Ko S C, Cha S H, Heo S J, Lee S H, Kang S M, Jeon Y J. 2011. Protective effect of *Ecklonia cava* on UVB-induced oxidative stress: in vitro and in vivo zebrafish model. *Journal of Applied Phycology*, **23**(4): 697-708.

Lopes G, Barbosa M, Vallejo F, Gil-Izquierdo Á, Andrade P B, Valentão P, Pereira D M, Ferreres F. 2018. Profiling phlorotannins from *Fucus* spp. of the northern Portuguese coastline: chemical approach by HPLC-DAD-ESI/MS^n and UPLC-ESI-QTOF/MS. *Algal Research*, **29**: 113-120.

Milledge J J, Nielsen B V, Bailey D. 2016. High-value products from macroalgae: the potential uses of the invasive brown seaweed, *Sargassum muticum*. *Reviews in Environmental Science and Bio/Technology*, **15**(1): 67-88.

Montero L, Sánchez-Camargo A P, García-Cañas V, Tanniou A, Stiger-Pouvreau V, Russo M, Rastrelli L, Cifuentes A, Herrero M, Ibáñez E. 2016. Anti-proliferative activity and chemical characterization by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry of phlorotannins from the brown macroalga *Sargassum muticum* collected on North-Atlantic coasts. *Journal of Chromatography A*, **1428**: 115-125.

Namvar F, Mohamad R, Baharara J, Zafar-Balanejad S, Fargahi F, Rahman H S. 2013. Antioxidant, antiproliferative, and antiangiogenesis effects of polyphenol-rich seaweed (*Sargassum muticum*). *BioMed Research International*, **2013**: 604787.

Puspita M, Déniel M, Widowati I, Radjasa O K, Douzenel P, Marty C, Vandanjon L, Bedoux G, Bourgougnon N. 2017. Total phenolic content and biological activities of enzymatic extracts from *Sargassum muticum* (Yendo) Fensholt. *Journal of Applied Phycology*, **29**(5): 2 521-2 537.

Qiao D L, Hu B, Gan D, Sun Y, Ye H, Zeng X X. 2009. Extraction optimized by using response surface methodology, purification and preliminary characterization

of polysaccharides from *Hyriopsis cumingii*. *Carbohydrate Polymers*, **76**(3): 422-429.

Samarakoon K W, Lakmal H H C, Kim S Y, Jeon Y J. 2014. Electron spin resonance spectroscopic measurement of antioxidant activity of organic solvent extracts derived from the methanolic extracts of Sri Lankan thebu leaves (*Costus speciosus*). *Journal of the National Science Foundation of Sri Lanka*, **42**(3): 190-195.

Sanjeewa K K A, Fernando I P S, Kim E A, Ahn G, Jee Y, Jeon Y J. 2017. Anti-inflammatory activity of a sulfated polysaccharide isolated from an enzymatic digest of brown seaweed *Sargassum horneri* in RAW 264.7 cells. *Nutrition Research and Practice*, **11**(1): 3-10.

Sanjeewa K K A, Fernando I P S, Samarakoon K W, Lakmal H H C, Kim E A, Kwon O N, Dilshara M G, Lee J B, Jeon Y J. 2016a. Anti-inflammatory and anti-cancer activities of sterol rich fraction of cultured marine microalga *Nannochloropsis oculata*. *Algae*, **31**(3): 277-287.

Sanjeewa K K A, Kim E A, Son K T, Jeon Y J. 2016b. Bioactive properties and potentials cosmeceutical applications of phlorotannins isolated from brown seaweeds: a review. *Journal of Photochemistry and Photobiology B*: *Biology*, **162**: 100-105.

Singleton V L, Rossi J A Jr, 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, **16**(3): 144-158.

Song J H, Piao M J, Han X, Kang K A, Kang H K, Yoon W J, Ko M H, Lee N H, Lee M Y, Chae S, Hyun J W. 2016. Anti-wrinkle effects of *Sargassum muticum* ethyl acetate fraction on ultraviolet B-irradiated hairless mouse skin and mechanistic evaluation in the human HaCaT keratinocyte cell line. *Molecular Medicine Reports*, **14**(4): 2 937-2 944.

Tanniou A, Vandanjon L, Incera M, Leon E S, Husa V, Le Grand J, Nicolas J L, Poupart N, Kervarec N, Engelen A, Walsh R, Guerard F, Bourgougnon N, Stiger-Pouvreau V. 2014. Assessment of the spatial variability of phenolic contents and associated bioactivities in the invasive alga *Sargassum muticum* sampled along its European range from Norway to Portugal. *Journal of Applied Phycology*, **26**(2): 1 215-1 230.

Terme N, Boulho R, Kendel M, Kucma J P, Wielgosz-Collin G, Bourgougnon N, Bedoux G. 2017. Selective extraction of lipid classes from *Solieria chordalis* and *Sargassum muticum* using supercritical carbon dioxide and conventional solid-liquid methods. *Journal of Applied Phycology*, **29**(5): 2 513-2 519.

Wijesinghe W A J P, Kim E A, Kang M C, Lee W W, Lee H S, Vairappan C S, Jeon Y J. 2014. Assessment of anti-inflammatory effect of 5β-hydroxypalisadin B isolated from red seaweed *Laurencia snackeyi* in zebrafish embryo *in vivo* model. *Environmental Toxicology and Pharmacology*, **37**(1): 110-117.

Xi L S, Mu T H, Sun H N. 2015. Preparative purification of polyphenols from sweet potato (*Ipomoea batatas* L.) leaves by AB-8 macroporous resins. *Food Chemistry*, **172**: 166-174.

Yang E J, Ham Y M, Lee W J, Lee N H, Hyun C G. 2013. Anti-inflammatory effects of apo-9'-fucoxanthinone from the brown alga, *Sargassum muticum*. *DARU Journal of Pharmaceutical Sciences*, **21**: 62.

Yoon W J, Ham Y M, Lee W J, Lee N H, Hyun C G. 2010. Brown alga *Sargassum muticum* inhibits proinflammatory cytokines, iNOS, and COX-2 expression in macrophage RAW 264.7 cells. *Turkish Journal of Biology*, **34**: 25-34.

Zhang R, Yuen A K L, Magnusson M, Wright J T, de Nys R, Masters A F, Maschmeyer T. 2018. A comparative assessment of the activity and structure of phlorotannins from the brown seaweed *Carpophyllum flexuosum*. *Algal Research*, **29**: 130-141.